Chapter 6

Summary and concluding remarks

To reach their site of action, extracellular proteins need to be transported across one or more membranes. This process is called protein translocation. In bacteria, the main barrier for protein translocation is the cytoplasmic membrane. All bacteria contain a specialized system to translocate protein across this barrier, and this system is termed the Sec-system. Over the last two decades, the Sec-system of the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis* has been studied in detail. The essential subunits of the secretion system have been found in both groups of bacteria (Suh *et al*., 1990; Overhoff *et al*., 1991; Brundage *et al*., 1992; Jeong *et al*., 1993). This has led to the conception that Gram-positive and Gram-negative bacteria secrete their proteins by similar mechanisms. On the other hand, complementation studies indicate that most components of the protein secretion system cannot be exchanged functionally between organisms (Nakamura *et al*., 1990; Suh *et al*., 1990; Takamatsu *et al*., 1994; Klein *et al*., 1994; Bolhuis *et al*., 1998; van Wely *et al*., 1999). This indicates some kind of species specificity, but the molecular basis for this specificity is unknown. Therefore, a comparison of the Gram-negative and Gram-positive protein secretion pathways and an understanding of the basic mechanisms underlying protein translocation are necessary. The work described in this thesis deals with the identification of components that are involved in protein secretion by *B. subtilis*. Functional aspects of the *B. subtilis* Sec-system have been compared to that of *E. coli*.

Precursor proteins are synthesized at the ribosome as extended polypeptides with a cleavable amino-terminal signal sequence (for reviews see Simonen and Palva, 1993; Dalbey *et al*., 1997). Cytosolic chaperones act to prevent the tight folding or aggregation of these precursor protein prior to their translocation across the membrane. In the case of co-translational translocation, the site of synthesis of the precursor is brought into contact with the translocation channel and the membrane translocation of the precursor protein is coupled to its synthesis at the ribosome (for review see Rapoport *et al*., 1996). This process involves the signal recognition particle (SRP), a targeting factor that binds to the signal sequence and guides the ribosome-nascent chain complex to the translocation channel. In the case of post-translational translocation, the precursor protein is synthesized completely before it is translocated across the membrane. This requires that tight folding of the precursor protein in the cytosol is prevented, a role fulfilled by molecular chaperones such as GroEL, DnaK and SecB (for reviews see Beissinger and Buchner, 1998; Fink, 1999). Membrane translocation occurs through an aqueous channel composed of the integral membrane proteins SecY, SecE and SecG (Manting *et al*., 2000). The driving force for translocation is provided by the hydrolysis of ATP by the SecA protein, a precursor protein stimulated ATPase (for review see Driessen *et al*., 1995), and by the proton motive force across the membrane (Muren and Randall, 1985; Meens *et al*., 1993). The complex of SecA, SecY, SecE and SecG is termed translocase.

In vitro protein translocation by the *B. subtilis* translocase

To study the requirements for protein translocation by *B. subtilis*, an *in vitro* protein translocation system was developed. Since the early eighties such a system has been available for the *E. coli* translocase, but previous attempts to obtain an *in vitro* system for *B. subtilis* failed. By using a broad range proteinase-inhibitor cocktail and a *B. subtilis* strain deficient in alkaline and neutral proteases, proteolysis of SecY could be prevented and inverted membrane vesicles, capable of translocating precursor proteins, were obtained. It appears that previous attempts to isolate translocation-competent inverted membrane vesicles of *B. subtilis* may have failed due to the high protease susceptibility of SecY. The precursors of α-amylase, preAmyL, and of alkaline phosphatase, prePhoB, were obtained from *B. licheniformis* and *B. subtilis*, respectively. The precursor proteins were overproduced in *E. coli* while blocking protein translocation, isolated and purified, and radiolabeled in a denatured form. Consequently, an authentic *in vitro* translocation system for protein secretion in *B. subtilis* was established. Translocation of preAmyL requires SecA and is driven by ATP hydrolysis and the proton motive force (PMF). Thus, the experiments confirm earlier *in vivo* observations in which translocation was shown to be sensitive to azide (Meens *et al*., 1993; Klein *et al*., 1994) and uncouplers of the PMF (Muren and Randall, 1985; Meens *et al*., 1993; Nakane *et al.*, 1995). Moreover, the results also agree with the data obtained in the *E. coli in vitro* system (Lill *et al*., 1989; Yamada *et al*., 1989). *In vitro*, the *B. subtilis* SecA could be replaced for the heterologous *E. coli* SecA, although the latter does not complement conditionally lethal *B. subtilis* SecA mutants (Takamatsu *et al*., 1994). Possibly the SecA proteins of both organisms have specificities that only are overlapping.

Identification of a *B. subtilis* SecG homologue

Protein translocation in *E. coli* is mediated by a multisubunit membrane protein complex that consists of a peripheral ATPase SecA and a preprotein conducting channel with SecY, SecE, and SecG as subunits. In *B. subtilis*, SecA, SecY and SecE have been identified previously through genetic analysis (Nakamura *et al*., 1990; Sadaie *et al*., 1991; Jeong *et al*., 1993), but *B. subtilis* SecG was not identified in this way. In contrast to the other components of the translocase, SecG is not essential for viability, but deletion of its gene renders the growth of some strains of *E. coli* cold sensitive (Nishiyama *et al*., 1994). *In vitro*, SecG greatly enhances the efficiency of secretion (Nishiyama *et al*., 1993). Attempts to clone the *secG* gene of *B. subtilis* by hybridisation with an *E. coli* probe failed, nor was it possible to obtain the gene by functional complementation of an *E. coli secG* null strain. After the completion of the genome sequence of *B. subtilis* (Kunst *et al*., 1997), sequence comparison identified a potential homologue of SecG, termed YvaL. Due to its weak homology it was necessary to characterize this protein further. The deletion of the *yvaL* gene from the *B. subtilis* chromosome and its effect on growth and protein secretion is descibed in this thesis.

A chromosomal disruption of the *yvaL* gene in *B. subtilis* resulted in a mild and incomplete cold-sensitivity of growth, and caused a secretion defect of some proteins at a lower temperature. The cold-sensitive phenotype of the *yvaL* deletion mutant became more evident after high-level production of pre-α-amylase. Experiments with a *secDF* null strain of *B. subtilis* have shown that, in analogy to SecG, SecDF is not essential for viability at 37ºC (Bolhuis *et al*., 1998). Under conditions of pre-α-amylase production, the growth defect of the *yvaL* null strain could be restored by expression of either the *B. subtilis* YvaL or the *E. coli* SecG. In contrast, YvaL could not complement the cold-sensitive growth defect of an *E. coli secG* deletion mutant. *In vitro*, the translocation of prePhoB strongly depends on the presence of YvaL. This was tested in membrane vesicles derived from an *E. coli* strain that overproduced the *B. subtilis* SecYE proteins alone or together with YvaL. The data firmly identify YvaL as a Gram-positive SecG homologue, and also explain why previous attempts to clone the gene by complementation and hybridisation have failed. Using the YvaL sequence, various other Gram-positive SecG homologues could be identified in databases.

Species-specificity is present in all components of the translocase

With the identification of the *Bacillus* SecG protein, overproduction of the entire integral membrane domain of the translocase in *E. coli* could be attempted. This can be used to establish a stable *in vitro* system without the proteolytic background of *B. subtilis*, and in addition allows for subunit swapping experiments, yielding hybrid translocases of *E. coli* and *B. subtilis*. Subunit swapping experiments can be used to study the host-specific functions of the various subunits of the translocase. All homologous and hybrid SecYEG complexes could be overexpressed at high levels, indicating that the heterologous SecE and SecY proteins are capable of forming a complex that protects SecY against degradation by FtsH (Taura *et al*., 1993; Kihara *et al*., 1995). It was not possible to overexpress the *B. subtilis* SecY in the absence of SecE, confirming previous observations with the *E. coli* protein. The translocation activity of the hybrid complexes varied depending on the precursor protein studied. The precursor of *E. coli* outer membrane protein A, proOmpA, was efficiently translocated by the membranes supplemented with the *E. coli* SecA, provided that the *E. coli* SecY was present. On the other hand, *B. subtilis* prePhoB was translocated only by translocase complexes composed of homologous subunits, *i.e.*, either the complete *E. coli* or *B. subtilis* SecYEG-SecA complex. These data indicate that each of the subunits contributes in an exclusive way to the specificity and functionality of the translocase, and suggest that fine-tuning of interactions can be critical for the catalytic activity. A possible explanation may be found in the formation of an active translocase complex. In *E. coli* this requires the ATP and precursor protein-dependent recruitment of four SecYEG complexes, that subsequently assemble into a large protein-conducting channel (Manting *et al*. 2000). It may well be possible that precursors differ in their ability to trigger the homologous and heterologous SecA proteins for this event, whereas the functionality of the complex may critically depend on specific interactions between the SecYEG subunits. Presumably, one or more of these interactions are compromised in some of the hybrid translocases.

Binding studies further showed that the *E. coli* SecA binds both the *E. coli* and *B. subtilis* SecYEG complex with high affinity. Such high affinity binding could not be demonstrated for the *B. subtilis* SecA, even though the enzyme is fully active in translocation. Apparently, an excess of SecA can overcome a strict requirement for high affinity binding of SecA to the SecYEG complex.

Absence of a SecB paralogue in *B. subtilis*

In *E. coli* and some other Gram-negative bacteria, the secretion-dedicated chaperone SecB interacts with the carboxy-terminus of SecA to target a subset of secretory proteins to the translocase (Fekkes *et al*., 1999). Various cloning strategies to obtain a *B. subtilis* SecB homolog have failed in the past, and with the availability of the complete sequence of the *B. subtilis* genome, it has become apparent that this organism lacks a protein with significant homology to SecB. Nevertheless, the presence of the highly conserved carboxyl-terminus in the *B. subtilis* SecA, which functions as a SecB binding domain in *E. coli*, suggests that a functional analogue might be present. Deletion of the carboxyl terminus of the chromosomal copy of the *B. subtilis* SecA yielded cells that are normally viable and secretion proficient. Analysis of the protein profile of secreted proteins suggested a mild secretion defect. However, semi-quantitative RT-PCR demonstrated that this apparent secretion defect was due to a lowered expression level of the respective proteins. The carboxyl terminus of the *B. subtilis* SecA was fused to glutathione S-transferase (GST) and used to screen for cytosolic proteins that bind to this domain. The fusion protein specifically bound MrgA, a protein that has been reported to function in relation to oxidative stress (Chen and Helmann, 1995). Deletion of the *mrgA* gene from the chromosome, however, did not result in a secretion defect nor did it induce an oxidative stress response. Based on these results, it was concluded that the carboxyl terminus of the *B. subtilis* SecA is not essential for secretion and viability. In contrast, in *E. coli* the carboxyl-terminus of SecA is essential for viability (Breukink *et al*., 1995). Since other studies sofar have not indicated the involvement of specific chaperones, protein secretion in *B. subtilis* may either occur independently of such proteins, or the chaperone activity may be catalyzed by other components of the general protein secretion pathway such as SecA and SRP.

Final remarks

The central components of the protein translocation system of Gram-negative and Gram-positive bacteria show a high degree of conservation, suggesting similar functions and working mechanisms. Several studies, however, indicate that the components bearing the most fundamental properties have evolved to adapt to specific needs and cannot be functionally exchanged. Our studies indicate that this incompatibility cannot be attributed to a single component, but arises from the complete system. The main differences

between *B. subtilis* and *E. coli* seem to be contained in the precursor protein targeting pathway that lead the proteins to the translocase. *B. subtilis* contains a more developed SRP as compared to *E. coli*, and this complex seems to be involved in the post-translational as well as co-translational targeting of secretory proteins to the translocase. In contrast, the *E. coli* SRP is thought to be involved in targeting of inner membrane proteins to the translocase, whereas SecB mediates the targeting of secretory proteins. Future work may yield further detailed insight in how the Gram-positive protein secretion system is organised and may facilitate the exploitation of these organisms in the high level production of heterologous proteins.